Cells and method for fermentatively preparing R-α-lipoic acid

The invention relates to cells and a method for fermentatively preparing R-α-lipoic acid.

In a large number of prokaryotes and eukaryotes, R-α-lipoic acid is an essential cofactor of certain multienzyme complexes. In these complexes, the R-α-lipoic acid is in each case covalently bound, by its carboxyl group, to the ε-amino group of a specific lysine residue of the corresponding enzyme, thereby forming what is termed a lipoamide. In this way, R-α-lipoic acid is a part of the E2 subunit of pyruvate dehydrogenase (PDH) [EC 2.3.1.12] or of α-ketoglutarate dehydrogenase (KGDH) [EC 2.3.1.61] and, in this location, plays a crucial role, as redox partner and acyl group-transferring agent, in the oxidative decarboxylation of α-keto acids. In addition, R-α-lipoic acid functions as an aminomethyl carrier in glycine cleavage enzyme systems.

The α-keto acid dehydrogenase which is best characterized physiologically and genetically is the *Escherichia coli* pyruvate dehydrogenase multienzyme complex. The three subunits E1 (pyruvate dehydrogenase), E2 (dihydrolipoamide acetyltransferase) and E3 (dihydrolipoamide dehydrogenase) are encoded by an operon consisting of the genes *aceE*, *aceF* and *lpd* and form a multienzyme complex, which is composed of 24 E1, 24 E2 and 12 E3 subunits. In this connection, the 24 E2 subunits form the core of the complex. The PDH E2 monomer (E2p) in turn has a modular structure composed of different domains which are connected to each other by way of flexible linker regions (see fig. 1). The N terminus of the protein contains three so-called lipoyl domains which are in each case composed of approx. 80 amino acid residues, with each of these

domains being able to bind precisely one molecule of R-α-lipoic acid as described above. These three PDH lipoyl domains exhibit a sequence identity with each other which is in each case very high (> 66%). The small central E3 binding domain is connected to the N-terminal region of the protein, with this binding domain in turn being connected to the C-terminal region which contains the catalytic domain (acetyltransferase).

The E2 subunit of α-ketoglutarate dehydrogenase (E2o) is encoded by the *sucB* gene and also has a modular construction; however, in contrast to the PDH E2 protein, it only possesses one lipoyl domain. While, at only about 22%, the sequence of the E2o lipoyl domain exhibits a relatively weak identity with the E2p lipoyl domains, the spatial structures of the lipoyl domains of the two E2 proteins are very similar (Reche and Perham, 1999, EMBO J. 18: 2673-2682).

The biotinyl domain of the biotin carboxyl carrier protein (BCCP) is also a protein which is only moderately homologous with the lipoyl domains of E2 proteins as far as its sequence is concerned but which is likewise structurally very similar. BCCP is normally biotinylated posttranslationally by the biotinyl protein ligase BirA at a specific lysine residue. However, there exist a variety of specifically mutated BCCP variants which can also be lipoylated, alternatively or even exclusively, by a lipoyl protein ligase at this specific lysine residue (Reche and Perham, 1999, EMBO J. 18: 2673-2682).

α-Lipoic acid is an optically active molecule having a chiral center on the carbon atom C6. In this connection, the R configuration of α-lipoic acid constitutes the naturally occurring enantiomer. It is only this form which exhibits physiological activity as a cofactor of the corresponding enzymes. α-Lipoic acid

can be present both in an oxidized form (5-[1,2]-dithiolan-3-yl-pentanoic acid) and in a reduced form (6,8-dimercaptooctanoic acid). In that which follows, the term "α-lipoic acid" is to be understood as meaning both forms as well as the respective salts of α-lipoic acid such as the calcium salt, potassium salt, magnesium salt, sodium salt or ammonium salt.

The biosynthesis of R-α-lipoic acid has been investigated intensively in the bacterium *Escherichia coli* in particular (see fig. 2). In this bacterium, octanoic acid which is bound covalently to the acyl carrier protein (ACP) serves as a specific precursor in lipoic acid synthesis. In a complex reaction, two sulfur atoms are transferred to the octanoic acid which has been activated in this way (octanoyl-ACP), resulting in the formation of R-α-lipoyl-ACP. This reaction is catalyzed by lipoic acid synthase [EC 2.8.1.-], which is the product of the *lipA* gene. It is ultimately the amino acid L-cysteine which serves as the sulfur donor in this reaction. The subsequent transfer of the R-α-lipoic acid of R-α-lipoyl-ACP to the E2 subunit of the α-keto acid dehydrogenases is catalyzed by lipoyl protein ligase B [EC 6.-.-.-], i.e. the *lipB* gene product, without, however, R-α-lipoyl-ACP or R-α-lipoic acid appearing as free intermediates in this connection (Miller et al., 2000, Biochemistry 39:15166-15178).

However, *E. coli* can also take up free R-α-lipoic acid from the surrounding medium and use it for forming functional α-keto acid dehydrogenases. For this, R-α-lipoic acid is initially activated by ATP to give R-α-lipoyl-AMP and then transferred to the corresponding enzyme subunits (see fig. 3). Both activities are catalyzed by lipoyl protein ligase A [EC 6.-.-.-], which is the product of the *lplA* gene. However, this lplA activity is not essential for wild-type strains of *E. coli* when endogenous lipoic acid synthesis and the transfer of the lipoyl group take place by way of the

LipA/LipB pathway. Thus, *lplA* mutants which no longer possess any detectable lipoyl protein ligase A activity but whose phenotype cannot be distinguished from that of a wild-type cell under normal growth conditions have, for example, been described (Morris et al., 1995, J. Bacteriol. 177: 1-10).

Little is known about the biosynthesis of R-α-lipoic acid in eukaryotes. However, it is presumed that the synthesis of R-α-lipoic acid, and its transfer to the corresponding enzymes, take place in the mitochondria of eukaryotic cells in a similar manner to that in bacteria.

In addition to its relevance as an essential constituent of enzymes playing a central role in the metabolism, the importance of α-lipoic acid for pharmacotherapy and for foodstuff supplementation (nutraceutical) was recognized at an early stage: because of its two thiol groups, α-lipoic acid possesses pronounced activity as an antioxidant and can therefore protect the body from harmful processes which are induced by oxidative stress. In addition, because of its property as a powerful reducing agent, α-dihydrolipoic acid, i.e. the reduced form of α-lipoic acid, is able to directly or indirectly regenerate other natural antioxidants in the body, such as ascorbic acid or α-tocopherol, which are oxidized or else replace these antioxidants when they are deficient. Accordingly, α-lipoic acid is of central importance in the interplay involving ascorbic acid, α-tocopherol and glutathione, i.e. what is termed the "antioxidant network". In addition, α-lipoic acid is used for preventing and controlling type II diabetes mellitus and its sequelae such as polyneuropathy, cataract or cardiovascular diseases.

The different biological activities of the two enantiomers of α-lipoic acid are currently the subject

of intensive investigations, in connection with which, however, it is becoming increasingly clear that using the pure R enantiomer of α-lipoic acid has distinct advantages over using the S form. Thus, *in-vitro* experiments have shown that only natural R-α-lipoic acid leads to the formation of functional α-keto acid dehydrogenases. By contrast, the S enantiomer even had an inhibitory effect on the stimulation of enzyme activity by R-α-lipoic acid. The reduction of α-lipoic acid, and, as a result, the regeneration of the antioxidatively active α-dihydrolipoic acid, in the mitochondria is of essential importance for the cell. The mitochondrial NADH-dependent lipoamide reductase in mammals exhibits an activity which is almost 20-fold higher with the R enantiomer than with the S form. Furthermore, R-α-lipoic acid has a markedly more powerful effect than the S enantiomer on the insulin-mediated glucose uptake and glucose metabolism of skeletal muscle cells in insulin-resistant rats. In addition, the R form exhibited an anti-inflammatory effect in animal experiments whereas the S form had, if anything, an analgesic effect. In order to avoid undesirable side-effects, therefore, it is in each case extremely desirable only to administer α-lipoic acid in the enantiomerically pure form.

At present, α-lipoic acid is only prepared industrially by means of chemical methods, with the racemate, composed of the R and S forms, always being formed as the end product (Yadav et al., 1990, J. Sci. Ind. Res. 49: 400-409). A variety of methods have been developed for isolating enantiomerically pure R-α-lipoic acid. For example, the racemate of α-lipoic acid or of one of the synthesis intermediates can either be resolved chemically using chiral auxiliary substances (Walton et. al, 1954, J. Amer. Chem. Soc. 76: 4748; DE 4137773) or enzymatically (Adger et al., 1995, J. Chem. Soc., Chem. Commun.: 1563-1564). In other methods, a racemate is not formed, due to an enantioselective synthesis

step, with it being possible for the new chiral center to be introduced either chemically (DE 3629116; DE 19533881; Bringmann et al., 1999, Z. Naturforsch. 54b: 655-661; DE 10036516) or by means of a stereospecific biotransformation using microorganisms (Gopalan and Jacobs, 1989, Tetrahedron Lett. 30: 5705-5708; Dasaradhi et al., 1990, J. Chem. Soc., Chem. Commun.: 729-730; DE 10056025). Other processes in turn start the chemical synthesis of enantiomerically pure α-lipoic acid using a naturally occurring chiral starting compound such as S-maleic acid or D mannitol (Brookes and Golding, 1988, J. Chem. Soc. Perkin Trans. I: 9-12; Rama Rao et al., 1987, Tetrahedron Lett. 28, 2183-2186). Because of what are in some cases elaborate synthesis steps, low yields and high material costs, none of the known methods for preparing enantiomerically pure R-α-lipoic acid is at present economical.

Nowadays, many low molecular weight natural products, such as antibiotics, vitamins or amino acids, are frequently prepared industrially by means of a fermentative method using different strains of microorganisms. The patent applications at the German Patent and Trademark Office having the reference numbers 10235270, 10245993 and 10258127 describe both different cells which secrete enantiomerically pure R-α-lipoic acid and methods in which enantiomerically pure R-α-lipoic acid is produced exclusively in a fermentation process. In this connection, use is made of cells which either overexpress a lipoic acid synthase gene or a lipoyl protein ligase B gene, individually or in combination, or exhibit reduced lipoyl protein ligase A activity. The amount of enantiomerically pure R-α-lipoic acid which is produced using these cells is still only very limited, which means that these fermentative methods are at present still not able to compete with chemical synthesis.

It is only in rare cases that a single genetic manipulation of a wild-type strain, as carried out in the course of what is termed metabolic engineering, leads to the overproduction of the desired compound on an adequate scale. Instead, a combination of several selective genetic manipulations, frequently also supplemented with classical mutagenesis/screening approaches, is necessary to achieve this.

Accordingly, the object of the present invention is to provide efficient cells which secrete enantiomerically pure R-α-lipoic acid into a culture medium.

This object is achieved by means of a cell which secretes enantiomerically pure R-α-lipoic acid into a culture medium, characterized in that it possesses a lipoyl protein ligase B activity which is elevated as compared with that of a wild-type strain and, at the same time, exhibits a concentration of a lipoylatable polypeptide which is elevated as compared with that of the wild-type strain.

Physiological and biochemical data indicate that lipoic acid is almost exclusively present in bound form in wild-type cells since the synthesis of R-α-lipoic acid takes place completely in protein-bound form (cf. fig. 1) (Herbert and Guest, 1975, Arch. Microbiol. 106: 259-266; Miller et al., 2000, Biochemistry 39: 15166-15178). Astonishingly, augmenting the lipoyl protein ligase B activity of a wild-type cell is sufficient to cause R-α-lipoic acid to be secreted into the culture medium (DE 10245993). Within the context of this invention, it has now been found, completely surprisingly, that augmenting the lipoyl protein ligase B activity in combination with increasing the intracellular concentration of a lipoylatable polypeptide leads to a markedly increased accumulation of enantiomerically pure R-α-lipoic acid in the culture medium of these cells.

Normally, all the lipoyl-binding sites of the E2 proteins (specific lysine residues) in a wild-type *E. coli* strain are saturated with R-α-lipoic acid, i.e. modified with lipoic acid (Packman et al., 1991, Biochem. J. 277: 153-158), since the *de novo* synthesis of the R-α-lipoic acid and the subsequent transfer of R-α-lipoyl-ACP to the corresponding lipoyl domains are well matched to each other. The increased presence of a lipoylatable polypeptide in a cell now has a variety of consequences:

- Unmodified, i.e. non-lipoylated lipoyl acceptor proteins accumulate since the lipoic acid synthesis capacity of the cell is no longer adequate for completely loading all the potentially lipoylatable polypeptides (Miles and Guest, 1987, Biochem. J. 245: 869-874; Ali and Guest, 1990, Biochem. J. 271: 139-145).
- Cells containing an elevated concentration of unmodified lipoyl acceptor proteins can take up externally supplied R-α-lipoic acid, and bind it to lipoyl acceptor proteins, to an increased extent as compared with wild-type cells (Morris et al., 1995, J. Bacteriol. 177: 1-10).
- In the case of a lipoic acid-producing strain, the excretion of R-α-lipoic acid is drastically reduced (see strain W3110Δ*lplA* pGS331 in example 3), probably because the R-α-lipoic acid which is synthesized *de novo* can now be linked to the lipoyl acceptor proteins which are available in increased quantity, resulting in excretion being prevented.

In accordance with these findings, the skilled person would a priori assume that the increased presence of an unmodified lipoylatable polypeptide in the cell would reduce the excretion of R-α-lipoic acid even in the case of other lipoic acid-producing strains, such as the strain W3110 pBAD-lipB, which exhibits elevated lipoyl protein ligase B activity (DE 10245993). While

it is true that, as a result of overexpressing the *lipB* gene, the cells according to the invention possess an elevated lipoyl protein ligase B activity, the simultaneous provision of unloaded lipoylatable polypeptides would ensure that sufficient substrate was present for the lipoyl protein ligase reaction, such that the entire R-α-lipoic acid which was formed *de novo* would be able to be linked to these proteins intracellularly. Nevertheless, R-α-lipoic acid is astonishingly secreted to an increased extent under these conditions.

Within the context of this invention, the lipoyl protein ligase B activity is preferably to be understood as meaning the lipoyl protein ligase activity of a cell which is encoded by the *lipB* gene and which has a strict preference for R-α-lipoyl-ACP over free R-α-lipoic acid as the substrate (see fig. 2).

Within the meaning of the present invention, a lipoyl protein ligase B activity which is elevated as compared with that of a wild-type strain is preferably to be understood as meaning that this activity is increased by at least a factor of 2, preferably by at least a factor of 5.

The *lipB* gene is preferably a gene which has the sequence SEQ ID NO: 1 and which encodes a protein having the sequence SEQ ID NO: 2, or a gene which encodes a functional variant of the *lipB* gene product, which variant has a sequence identity with SEQ ID NO: 2 of greater than 35%.

Particular preference is given to functional variants of the *lipB* gene product which have a sequence identity with SEQ ID NO: 2 of greater than 55%, with very particular preference being given to functional variants which have a sequence identity with SEQ ID NO: 2 of greater than 80%.

Within the meaning of the present invention, a functional variant of the *lipB* gene product is preferably to be understood as meaning a protein which has an amino acid sequence which is derived, by means of the deletion, insertion or substitution of nucleotides, from the sequence depicted in SEQ ID NO: 1, with the enzymic activity of the lipoyl protein ligase B which is encoded by this gene being preserved.

Within the context of this invention, a "lipoylatable polypeptide" is to be understood as meaning peptides or proteins to which at least one molecule of R-α-lipoic acid can be bonded covalently. In this connection, this bonding is preferably effected between the carboxyl group of the R-α-lipoic acid and the ε-amino group of a lysine residue of the polypeptide, resulting in the formation of a lipoamide. In the cell, the formation of such a lipoamide bond is preferably catalyzed by a lipoyl protein ligase.

Within the meaning of the present invention, a concentration of a lipoylatable polypeptide which is elevated as compared with that in the wild-type strain is preferably to be understood as meaning that the quantity of this polypeptide in a cell is increased at least by a factor of 2, preferably at least by a factor of 5.

A gene which encodes a lipoylatable polypeptide preferably comprises a DNA fragment which has the sequence SEQ ID NO: 3 and which encodes a polypeptide having the sequence SEQ ID NO: 4, or a DNA fragment which encodes a functional variant of this polypeptide, which functional variant has a sequence identity with SEQ ID NO: 4 of greater than 20%.

Particular preference is given to genes which encode variants of a lipoylatable polypeptide which have a sequence identity with SEQ ID NO: 4 of greater than

40%, with very particular preference being given to genes which encode polypeptide variants which have a sequence identity with SEQ ID NO: 4 of greater than 70%.

It is also possible to use, as alternatives for genes which encode a lipoylatable polypeptide, alleles of genes which originally encoded a biotinylatable polypeptide (e.g. BCCP) but which can now, after minor sequence variation, also be lipoylated (e.g. BCCP-DASMEP). Such a gene comprises a DNA fragment which has the sequence SEQ ID NO: 5 and which encodes a polypeptide having the sequence SEQ ID NO: 6, or a DNA fragment which encodes a functional variant of this polypeptide which has a sequence identity with SEQ ID NO: 6 of greater than 75%.

Within the meaning of the present invention, a functional variant of a lipoylatable polypeptide is to be understood as meaning a protein having an amino acid sequences which is derived, by means of the deletion, insertion or substitution of nucleotides, from the sequences depicted in SEQ ID NO: 3 or SEQ ID NO: 5, with the property of being able to be lipoylated by a lipoyl protein ligase being preserved.

In the present invention, all the values for the sequence identity of DNA sequences and amino acid sequences refer to results which are obtained using the BESTFIT algorithm (GCG Wisconsin Package, Genetics Computer Group (GCG) Madison, Wisconsin).

Cells according to the invention which, in addition to an augmented lipoyl protein ligase B activity, exhibit a concentration of a lipoylatable polypeptide which is elevated as compared with that of the wild type can be produced using standard techniques of molecular biology.

Methods for increasing the activity of lipoyl protein ligase B in a cell are known in the prior art and are described, for example, in patent application DE 10245993, which, in this respect, is hereby expressly incorporated by reference.

The level of a lipoylatable polypeptide in cells according to the invention is increased, for example, by the expression of a gene which encodes a lipoylatable polypeptide being increased. In this connection, the copy number of such a gene can be increased in the cells and/or suitable promoters can be used to increase the expression of this gene.

In this connection, "increased expression" is preferably to be understood as meaning that the expression of the gene which encodes a lipoylatable polypeptide is increased at least by a factor of 2, preferably at least by a factor of 5 as compared with that of the gene in the respective wild-type cell from which this gene was obtained.

Methods known to the skilled person can be used to increase the copy number, in cells, of the gene which encodes a lipoylatable polypeptide. Thus, such a gene can, for example, be cloned into plasmid vectors having multiple copy numbers per cell (e.g. pUC19, pBR322 or pACYC184, or derivatives thereof, in the case of *Escherichia coli*) and these vectors can be introduced into the cells. Alternatively, such a gene can be integrated several times into the chromosome of the host organism. The integration methods which can be used are the known systems employing temperate bacteriophages, integrative plasmids or integration by way of homologous recombination (e.g. Hamilton et al., 1989, J. Bacteriol. 171: 4617-4622).

Preference is given to increasing the copy number by cloning a gene encoding a lipoylatable polypeptide into

a plasmid vector under the control of a promoter. Particular preference is given to increasing the copy number by cloning such a gene into plasmid vectors belonging to the pUC family or to the pBR322 family, for example ptac85 (Ali and Guest, 1990, Biochem. J. 271: 139-145).

While the natural promoter and operator regions of a gene for a lipoylatable polypeptide can serve as the control region for expressing this gene, it is also possible, in particular, to increase the expression of such a gene by means of using other promoters. Appropriate promoter systems in the case of *Escherichia coli*, such as the constitutive promoter of the *gapA* gene or the inducible lac, tac, trc, λ, ara or tet promoters, are known to the skilled person (Makrides S.C., 1996, Microbiol. Rev. 60: 512-538). Constructs which contain a gene for a lipoylatable polypeptide under the control of one of the above-mentioned promoters can be used on plasmids or chromosomally, in a manner known per se.

In a preferred embodiment, cells according to the invention harbor a plasmid which contains a gene for a lipoylatable polypeptide under the control of a promoter which is selected from the *gapA*, lac, tac, trc, λ, ara or tet promoter group. In a particularly preferred embodiment, this gene is under the control of the isopropyl-β-D-thiogalactoside/*lacI*-regulatable tac promoter.

In addition, elevated expression can be achieved by translation start signals, such as the ribosome binding site or the start codon of the gene, being present in optimized sequence on the given construct or by codons which are rare in accordance with the codon usage being replaced by codons which occur more frequently.

A gene which encodes a lipoylatable polypeptide is, for

example, cloned into plasmid vectors by amplifying the gene by means of the polymerase chain reaction using specific primers which cover the complete gene or at least the part of the gene which encodes a lipoylatable polypeptide (e.g. the lipoyl domain of a protein), and then ligating it to vector DNA fragments.

It is possible to use standard methods of molecular biology to generate a gene for a lipoylatable hybrid polypeptide which is composed, for example, of parts of two different lipoyl domains of the E2 protein, and to clone the gene into a plasmid vector, with these procedures being described, for example, in Miles and Guest (1987, Biochem. J. 245: 869-874).

The preferred vectors to be used for cloning a gene which encodes a lipoylatable polypeptide are plasmids which already contain promoters for ensuring elevated expression, for example the heat-inducible $\lambda P_L P_R$ promoter or the isopropyl-β-D-thiogalactoside/*lacI*-regulatable synthetic tac promoter derived from *Escherichia coli*.

The abovementioned procedures which lead to the over-expression of a single gene can also be combined with each other in order to synchronously overexpress a *lipB* gene together with a gene which encodes a lipoylatable polypeptide. Thus, the two relevant genes can, for example, be present on different plasmids and the expression of the genes can in each case be under the control of different promoter systems. However, it is also possible for the two genes to be present as an artificial operon on the same plasmid and for the expression of the two genes consequently to be regulated synchronously by the same promoter. It is furthermore possible for the *lipB* gene and the gene for a lipoylatable polypeptide also to be located on the same plasmid with each gene being regulated by its own promoter. In this case, the two promoters can either

belong to the same type or belong to different types.

Plasmids which contain both a *lipB* gene and a gene for a lipoylatable polypeptide also form part of the subject matter of the present invention.

In a preferred embodiment, the two genes are present on the same plasmid, with each gene being under the control of its own isopropyl-β-D-thiogalactoside/*lacI*-regulatable tac promoter.

The invention consequently also relates to a plasmid which is characterized in that it carries a *lipB* gene and also a gene which encodes a lipoylatable polypeptide, in each case under the control of a promoter.

A customary transformation method (e.g. electroporation) is used to introduce the plasmids which contain a *lipB* gene and/or a gene for a lipoylatable polypeptide into a starting cell, with antibiotic resistance being used, for example, to select for plasmid-harboring clones.

The invention consequently also relates to a method for preparing a cell according to the invention, characterized in that a plasmid which contains a *lipB* gene and a plasmid which contains a gene for a lipoylatable polypeptide, or a plasmid according to the invention, is/are introduced into a starting cell.

Genes which encode lipoylatable polypeptides (e.g. *aceF*, *sucB*) and genes which are required for the *de-novo* synthesis of R-α-lipoic acid (e.g. *lipA*, *lipB*) have been identified in a large number of prokaryotic and eukaryotic cells or organisms. Cells according to the invention can consequently preferably be prepared from cells of prokaryotic or eukaryotic organisms which are themselves able to synthesize R-α-lipoic acid (starting cell), which are accessible to recombinant

methods and which can be cultured by fermentation. Plant or animal cells which can be propagated in cell culture are consequently also suitable for preparing cells according to the invention.

It is possible to use starting cells which have not previously been subjected to any manipulation for preparing cells according to the invention. However, it is furthermore also possible to combine the cells according to the invention with procedures which already lead to an improved production of R-α-lipoic acid. Thus, those cells which already possess an elevated lipoic acid synthase activity as a result of an increased expression of *lipA* gene and/or exhibit a lipoyl protein ligase A activity which is either only attenuated or, preferably, completely suppressed are, for example, particularly suitable. Methods for preparing cells which have an increased lipoic acid synthase activity and/or an attenuated lipoyl protein ligase A activity are described in patent applications DE 10235270 and DE 10258127.

The cells are preferably microorganisms such as yeast strains or bacterial strains. They are particularly preferably bacterial strains from the Enterobacteriaceae family, very particularly preferably strains of the species *Escherichia coli*.

The invention furthermore relates to a method for fermentatively preparing enantiomerically pure R-α-lipoic acid. This method is characterized in that a cell according to the invention is cultured in a culture medium, with the cell secreting enantiomerically pure R-α-lipoic acid into the culture medium and the enantiomerically pure R-α-lipoic acid being separated off from the culture medium.

The fact that R-α-lipoic acid is secreted out of the cells according to the invention and into the culture

medium enables the product to be isolated readily from the culture medium after the biomass has been separated off, i.e. without the cells having to be previously disrupted and/or without an elaborate and loss-associated hydrolytic step having to be used to cleave the R-α-lipoic acid from the carrier protein (ACP or the E2 subunit of the α-keto acid dehydrogenases) which is bound to it. The R-α-lipoic acid can be isolated from the culture medium using methods known to the skilled person, for example by centrifuging the cell-containing culture medium in order to separate off the cells and by subsequently extracting and/or precipitating the product.

In order to produce R-α-lipoic acid, the cells according to the invention are cultured in customary culture media, preferably in a minimal salt medium which is known from the literature (Herbert and Guest, 1970, Meth. Enzymol. 18A, 269-272).

In principle, any utilizable sugars, sugar alcohols or organic acids or their salts can be used as the carbon source. In this connection, preference is given to using aspartic acid, malic acid, succinic acid, pyruvic acid, fumaric acid, glutamic acid, glucose, glycerol or oxaloacetic acid. Succinic acid and oxaloacetic acid are particularly preferred. It is also possible to provide a combined feed consisting of several different carbon sources. It is furthermore possible to add short-chain fatty acids having a chain length of C2-C8, preferably having a chain length of C6-C8 (hexanoic acid or octanoic acid) to the medium, as specific precursors for the α-lipoic acid synthesis. In this connection, the concentration of the added carbon source is preferably 0.1-30 g/l.

The cells according to the invention are preferably incubated under aerobic culture conditions for a period of 16-150 h and in the region of the growth temperature

which is optimal for the given cells.

The preferred optimal temperature range is 15-55°C. A temperature of between 30 and 37°C is particularly preferred.

The R-α-lipoic acid which is produced in the method according to the invention is, for example, detected and quantified by means of a bioassay using an indicator strain (*lipA* mutant) which is auxotrophic for lipoic acid. This type of turbidimetric quantification of R-α-lipoic acid is known from the literature (Herbert and Guest, 1970, Meth. Enzymol. 18A, 269-272). However, the indicator strain W1485lip2 (ATCC 25645) which is used within the context of the present invention would also grow without any R-α-lipoic acid supplement if the medium also contained acetate and succinate in addition to glucose. In order to avoid a falsely positive growth of the indicator strain in the bioassay when determining the R-α-lipoic acid which has been produced, with the growth being caused, for example, by a charge of glucose and the acetate and succinate acids which have been secreted by the producer strain in addition to the R-α-lipoic acid, the R-α-lipoic acid producer is already preferably grown using succinate as the sole carbon source. This strain is supplemented with the culture supernatant from a cell growth in accordance with the invention; the growth of the indicator strain can then be used to determine the lipoic acid content in the culture medium.

The following examples serve to further clarify the invention. The bacterial strain *Escherichia coli* W3110/pKP560/pGS331, which was used for carrying out the examples, was deposited in the DSMZ (Deutsche Sammlung für Mikroorganismen und Zellkulturen [German Collection of Microorganisms and Cell Cultures] GmbH, D-38142 Braunschweig) under the number DSM 15661 in

accordance with the Budapest Treaty. The strain W3110Δ*lplA*, a mutant with a deletion in the *lplA* gene which encodes lipoyl protein ligase A, is described in patent application DE 10258127 from the same applicant. The plasmid pACYC184 is described in Chang and Cohen (1978, J. Bacteriol. 134: 1141-1156) while the *lipB* expression plasmid pBAD-lipB is described in patent application DE 10245993. The plasmid pGS331 (Ali et al., 1990, Biochem. J. 271: 139-145) was used for expressing the gene which encodes a lipoylatable polypeptide. In addition to the ampicillin resistance gene, this plasmid contains a subgene fragment of the *Escherichia coli aceF*-(E2p) gene which encodes a lipoyl domain and which is under the expression control of the tac promoter. The gene for this lipoyl domain is in this case a hybrid which is composed of the codons for amino acid residues 1-33 of the first lipoyl domain and amino acid residues 238-289 of the third lipoyl domain of the E2p protein.

Example 1: Constructing the *lipB* expression plasmid pKP560

The plasmid vector pACYC184 was first of all cut with the restriction endonuclease *AvaI* under the conditions specified by the manufacturer. The 5'-protruding ends of the restricted vector which were generated in this way were filled using the Klenow polymerase in accordance with the manufacturer's instructions, after which the vector was cut with the restriction endonuclease *ClaI* and then dephosphorylated by treating it with alkaline phosphatase. The whole restriction mixture was then separated electrophoretically in an agarose gel. The 2.8 kb DNA fragment, which also still contained the chloramphenicol resistance gene in addition to the p15A origin of replication, was then isolated from the agarose gel, and purified, using QIAquick gel extraction kit (Qiagen GmbH, Hilden) in accordance with the manufacturer's instructions. The *lipB* gene, under the control of the arabinose-inducible *ara-BAD* promoter

(pBAD), was isolated from the plasmid pBAD-lipB, which was first of all cut with the restriction endonuclease *XbaI* under the conditions specified by the manufacturer. The 5'-protruding ends of the restricted vector which were generated in this connection were then filled using the Klenow polymerase in accordance with the manufacturer's instructions, after which the vector was cut with the restriction endonuclease *ClaI*. The whole restriction mixture was then separated electrophoretically in an agarose gel. The 2 kb DNA fragment, which contained the regulatory sequences of the *E. coli* arabinose operon (*araC* gene, *araBAD* promoter region) as well as the *lipB* gene under the control of the *araBAD* promoter, was then isolated from the agarose gel, and purified, as described for the pACYC184 vector fragment.

The *araC*-pBAD-*lipB* fragment was ligated to the 2.8 kb pACYC184 vector fragment using T4 DNA ligase. *E. coli* cells of the strain DH5α were transformed with the ligation mixture by means of electroporation, in a manner known to the skilled person. The transformation mixture was then spread on LB-chloramphenicol agar plates (10 g of tryptone/l, 5 g of yeast extract/l, 10 g of NaCl/l, 15 g of agar/l, 20 mg of chloramphenicol/l) and the plates were incubated overnight at 37°C. The desired transformants were identified by restriction analysis after plasmid isolation had taken place using the GFX™ Micro Plasmid Prep kit (Amersham Biosciences GmbH, Freiburg). The vector which is obtained in this way is designated pKP560 (fig. 4).

Example 2: Preparing R-α-lipoic acid producers

The *lipB* overexpression plasmid pKP560 and/or the lipoyl domain plasmid pGS331 were transformed by electroporation into the *E. coli* strains W3110 and/or W3110Δ*lplA* and, following selection on LB agar plates containing 20 mg of chloramphenicol/l and/or 100 mg of ampicillin/l, the plasmids were reisolated from in each

case one of the transformants, cleaved with restriction endonucleases and checked. An analogous procedure was used in the case of the control plasmid pACYC184.

For joint overexpression of the *lipB* gene together with the lipoyl domain gene, the strains W3110 pKP560 and/or W3110Δ*lplA* pKP560 were transformed with plasmid pGS331 as described above and the resulting transformants were checked by means of restriction analysis.

Example 3: Producing R-α-lipoic acid fermentatively

The strains which were generated in example 2 as a result of transformation with the corresponding plasmids were used for the fermentative production of R-α-lipoic acid. As a preliminary culture for the production growth, 5 ml of liquid LB medium, which contained 100 mg of ampicillin/l and/or 20 mg of chloramphenicol/l for stabilizing plasmids, were first inoculated with the relevant strain and incubated at 37°C and 160 rpm for 16 h on a shaker. The cells were then harvested by centrifugation and washed twice with the corresponding volume of sterile saline (0.9% NaCl). Finally, 15 ml of BS medium (7·g of K_2HPO_4/l; 3 g of KH_2PO_4/l; 1 g of $(NH_4)_2SO_4$/l; 0.1 g of $MgSO_4 \times 7H_2O$/l; 0.5 g of Na_3 citrate $\times 3H_2O$/l; 1% acid-hydrolyzed caseine (vitamin-free); 13.5 g of Na_2 succinate $\times 6H_2O$/l; adjusted to pH 6.8 with HCl), which additionally contained 100 mg of ampicillin/l and/or 20 mg of chloramphenicol/l, were inoculated in a ratio of 1:100 with the cells which had been prepared in this way. The production cultures were incubated at 37°C and 160 rpm on a shaker. Expression of the lipoyl protein ligase B gene in the strains which harbored plasmid pKP560 was induced by adding 2 g of L-arabinose/l after approx. 4 h of incubation. At the same time, the expression of the E2 domain in the strains harboring plasmid pGS331 was also induced by adding 0.05 g of isopropyl-β-D-thiogalactoside/l. After 24 h of incubation, samples were removed and the cells were separated off

from the culture medium by centrifugation. The R-α-lipoic acid which was present in the culture medium was quantified using the known turbidimetric bioassay (Herbert and Guest, 1970, Meth. Enzymol. 18A: 269-272). Table 1 shows the contents of R-α-lipoic acid which were achieved in each respective culture supernatant after 24 h of incubation:

Table 1:

Strain	**R-α-lipoic acid [µg/l]**
W3110 pACYC184	0
W3110 pKP560	20
W3110 pKP560 pGS331	50
W3110 pGS331	0
W3110Δ*lplA* pACYC184	23
W3110Δ*lplA* pKP560	119
W3110Δ*lplA* pKP560 pGS331	220
W3110Δ*lplA* pGS331	2